Volume 336, number 2, 343–346 FEBS 13437 December 1993

Percoll purification of chromaffin granules inhibits their ability to take up and maintain calcium

Peter G. Jones, Joel Meneses, David M. Waisman*

MRC Signal Transduction Group, Department of Medical Biochemistry, University of Calgary Medical Sciences Centre, Alba, T2N 4N1, Canada

Received 19 October 1993; revised version received 9 November 1993

Secretory granules of the adrenal medulla have recently been shown to be able to sequester and release Ca^{2+}, in addition to their previously established role as carriers of secretory products. In order to study the ability of these or any other secretory granules to participate in intracellular calcium homeostasis, it is imperative that they should be free of other contaminating Ca^{2+} sequestering organelles, and that the Ca^{2+} uptake and release mechanisms of those granules should remain intact throughout any chosen purification procedure. We report here that chromaffin granules which were purified by the isopycnic gradient medium Percoll, or even incubated with it, showed an attenuated ability to sequester Ca^{2+}.

Chromaffin granule; Percoll purification gradient; Ca^{2+}-sequestration; Ca^{2+}-homeostasis; Metrizamide purification gradient

1. INTRODUCTION

Isolated chromaffin granules of the adrenal medulla display the ability to sequester Ca^{2+} [1] by the activity of a Na^+/Ca^{2+} exchange protein ([2] and Jones et al., Biochemistry, submitted) and maintain it by virtue of high capacity calcium binding protein within their lumen [3] and a boundary membrane. These properties taken together allow granules from adrenal medullary cells to accumulate up to 30 mM Ca^{2+} [4], some of which has been shown to be eliminated concomitantly with catecholamines during exocytosis [5] and therefore may represent a cellular mechanism for the elimination of Ca^{2+}[i] acquired during cellular stimulation. Two other observations have recently been made on the possible destiny of the Ca^{2+} sequestered by chromaffin granules. Firstly, it has been shown that inositol trisphosphate can induce a release of Ca^{2+} from these organelles [6], and secondly, we have recently demonstrated that an intracellularly localized member of the annexin family of Ca^{2+}-dependent, phospholipid-binding proteins, annexin VI, is also able to release sequestered Ca^{2+} from chromaffin granules (Jones et al., Biochemistry, submitted). These observations raise the possibility that the release and/or sequestration of chromaffin granule Ca^{2+} may contribute to the intracellular Ca^{2+} signal observed during stimulus-secretion coupling.

In order to study either the Ca^{2+} uptake or release mechanisms of these organelles without interference from other Ca^{2+} sequestering systems, such as cytosolic calcium binding proteins, endoplasmic reticulum and mitochondria, various purification techniques, mainly involving centrifugation have been employed to obtain pure secretory granules. Differential centrifugation has been used [2], but the purity of granules obtained by this method is not optimal, and while hyperosmotic sucrose gradients have also been used for granule purification [7], there are problems of granule lysis upon returning the granules to isotonicity [8], therefore effectively ruling out this isolation procedure for the production of intact granules. The remaining purification techniques, which use isopycnic gradients such as Percoll, Metrizamide, and Ficoll (e.g. [9,10,11]) can be utilized to produce intact chromaffin granules. We initially used Percoll-purified chromaffin granules to study the Ca^{2+} uptake and release mechanisms of chromaffin granules. Using one batch of Percoll we were able to produce granules which sequestered Ca^{2+}, however, subsequent batches of Percoll inhibited their ability to sequester, or maintain any sequestered Ca^{2+}, when compared with chromaffin granules produced by the Metrizamide purification method. Finally, chromaffin granules which were purified on Metrizamide density gradients lost both their ability to sequester Ca^{2+} and also their facility to maintain sequestered Ca^{2+} upon incubation with low concentrations of Percoll.

2. EXPERIMENTAL

2.1. *Crude granule preparation*

Bovine adrenal glands were obtained within 5 min of the animal's death and were transported to the laboratory on ice. All isolation procedures were performed at 4°C and all solutions were buffered with triethanolamine. Medullae were coarsely homogenized in 10 vols. of buffer A (300 mM sucrose, 5 mM EGTA, 25 mM HEPES, pH 7.5), in a Waring commercial food blender at the highest setting (3 × 2 s bursts, 1 min apart). This course tissue suspension was further dis-

*Corresponding author. Fax: (1) (403) 283 4841.

rupted by several strokes in a Dounce homogenizer, followed by centrifugation at 1,000 × *g* for 10 min. The supernatant was filtered through two layers of cheesecloth and further centrifuged (40 min, 10,000 × *g*). The upper layer of the pellet was washed away with buffer A, to partially remove mitochondrial contamination and the pellet was resuspended in 40 ml of buffer B (300 mM sucrose, 1 mM EGTA, 25 mM HEPES, pH 7.5). This crude granule fraction was processed by density gradient centrifugation, as follows.

2.2. *Percoll purification of chromaffin granules*

Granules were purified essentially as per the method of Meyer and Burger [12], with the following minor modifications. The Percoll gradient consisted of 3 × 12 ml of each 20%, 40% and 60% Percoll which contained 1 mM EGTA, pH 7.5, and were each adjusted to a final osmolality of 340 mOsm by the addition of sucrose. Each gradient was loaded with 7 ml of the crude granule suspension with centrifugation for 60 min at 25,000 × *g*. Purified granules, which collected at the 40%/60% interface, were washed twice in 100 ml of buffer B (10,000 × *g* for 40 min). The pellet was resuspended in sufficient buffer B, to produce a stock of 4–8 mg of granule protein/ml, and stored at 4°C until used. Granules were diluted to between 2 and 4 mg of granule protein/ml with buffer B prior to use.

2.3. *Metrizamide isolation of chromaffin granules*

Chromaffin granules were purified according to Morris and Schovanka [13] with modifications. 2 ml of crude granules were top-loaded onto a 400 mOsm sucrose/metrizamide density gradient consisting of 40% metrizamide at the top and ending with 100% metrizamide. The 100% component of the gradient contained 36 g of Metrizamide in 100 ml of buffer containing 1 mM EGTA and 25 mM HEPES, pH 7.5, and the 40% component was comprised of buffer B/100% Metrizamide 60:40 (v/v). After centrifugation (32,500 × *g*, 60 min), the middle of the three bands from each gradient were pooled and washed twice in 100 ml of buffer B by further centrifugation (10,000 × *g*, 40 min). The pellet was resuspended in the same buffer and stored at between 4 and 8 mg of granule protein/ml) at 4°C until used. Prior to use the granules were diluted with buffer B to between 2 and 4 mg of granule protein/ml.

2.4. *Ca^{2+} uptake*

Purified chromaffin granules at 0.5–1.0 mg/ml were incubated in a buffer containing 0.3 M sucrose, 0.5 mM EGTA, 25 mM HEPES, pH 7.5, and where indicated various concentrations of Percoll which had been dialyzed against 300 mM sucrose, 25 mM HEPES, pH 7.5. To initiate Ca^{2+} uptake, $CaCl_2$ (containing 10 cpm/pmol $^{45}Ca^{2+}$) was added to a final concentration of 0.65 mM and the reaction was allowed to progress for 45 min at 37°C. The reaction was terminated by filtration (nitrocellulose pore size 0.45 μm) and the granule sequestered $^{45}Ca^{2+}$ determined by liquid scintillation spectrometry. Ca^{2+} uptake data are expressed as nmol of Ca^{2+} per mg of granule protein.

2.5. *$^{45}Ca^{2+}$ release*

To determine Percoll-induced Ca^{2+} release from chromaffin granules, the granules were first $^{45}Ca^{2+}$ loaded as described above. To initiate $^{45}Ca^{2+}$ release, an aliquot of this mixture was diluted 1/1 into 0.3 M sucrose, 25 mM HEPES, pH 7.5, which contained where indicated, either 0.3 M sucrose, 25 mM HEPES, pH 7.5, [buffer C] as a control, or various concentrations of Percoll (equilibrated for 12 h against buffer C). The release reaction was terminated by filtration (nitrocellulose, pore size 0.45 μm) with 10 ml of buffer A and granule sequestered $^{45}Ca^{2+}$ was determined by liquid scintillation spectrometry. Release data are expressed as percentage of granule-sequestered $^{45}Ca^{2+}$, measured in the absence of added Percoll where % release = (cpm control granules − cpm test granules)/cpm control granules × 100.

2.6. *Determination of catecholamine content of isolated chromaffin granules*

Purified chromaffin granules were allowed to take up Ca^{2+} at 37°C for 45 min in a buffer containing 300 mM sucrose, 25 mM HEPES, pH 7.5, to which 150 μM $CaCl_2$ was added. 75 μl aliquots were incubated for 10 min with 25 μl of buffer C containing various amounts of Percoll (equilibrated against 300 mM sucrose, 25 mM HEPES, pH 7.5, for 12 h) to give the final concentrations of Percoll shown. Aliquots were centrifuged (10,000 × *g*, 40 min) and the pellet was disrupted in 25 mM MES buffer, pH 5.5, and the pellet content of catecholamines determined by the trihydroxyindole spectrophotometric assay [14] with modifications [15]. Samples were read at 412 nm excitation, 523 nm emission on a Perkin Elmer 650-10S fluorescence spectrophotometer.

2.7. *Miscellaneous*

Protein concentrations were determined according to Bradford [16], using BSA as a standard.

2.8. *Materials*

All chemicals were of analytical grade: HEPES and EGTA (acid form) from Sigma Chemical Co.; A23187 from Calbiochem; Triethanolamine from Fisher; Sucrose from BDH chemicals; Metrizamide from Accurate Chemicals and Scientific Corporation; $^{45}Ca^{2+}$ from Amersham; Metricel filters (0.45 μm) from Gelman Sciences; Filter Count Scintillation fluid from Packard, Illinois; Percoll from Pharmacia (batch numbers: QD11647, QA10474, QG11833). Dialysis tubing, Spectra/Por type 2 from Spectrum Medical Industries. Bovine adrenal glands were obtained from XL Beef Co., Calgary, Alberta.

3. RESULTS AND DISCUSSION

Chromaffin granules prepared by purification on Percoll density gradients showed a significant attenuation of $^{45}Ca^{2+}$ uptake when compared against those prepared by Metrizamide purification (Fig. 1). Given that such a dramatic difference in uptake capacity existed between granules prepared by these different methods, we considered the possibility that a physical interaction between the Percoll beads and chromaffin granules dur-

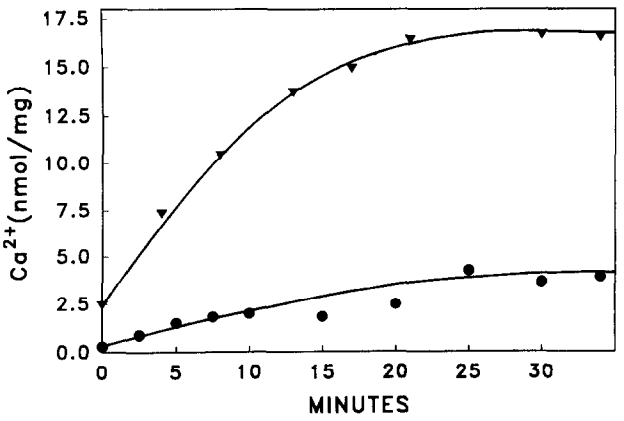

Fig. 1. Time course of Ca^{2+} uptake into chromaffin granules by Percoll and metrizamide prepared granules. Purified chromaffin granules, prepared by Metrizamide gradient (▽) and Percoll gradient (●) (see section 2) were incubated in a buffer containing 0.3 M sucrose, 0.5 mM EGTA and 25 mM HEPES, pH 7.5, at a final concentration of 0.5 mg/ml. To initiate Ca^{2+} uptake, $CaCl_2$ (containing 10 cpm/pmol $^{45}Ca^{2+}$) was added to a final concentration of 0.65 mM and the reaction was allowed to progress at 37°C. At the indicated times, aliquots were removed and accumulated $^{45}Ca^{2+}$ was determined by liquid scintillation spectrometry (section 2). Data shown are representative of three experiments.

ing centrifugation could alter their ability to sequester $^{45}Ca^{2+}$, or alternatively that there might be soluble contaminants in Percoll, which inhibit the Ca^{2+} sequestration mechanism of the granules. For example, the presence of between 50 to 100 mM extravesicular Na^+ has been clearly demonstrated to reverse the direction of Na^+/Ca^{2+} exchanger [4], and the presence of such a contaminant could explain the observed reduced ability of the granules to sequester $^{45}Ca^{2+}$. We tested both of these possibilities simultaneously, by incubating the granules with 25% Percoll which had been dialysed to remove potential soluble contaminants.

Chromaffin granules which had been Metrizamide purified and then incubated with 25% dialysed Percoll also showed a significant attenuation of $^{45}Ca^{2+}$ uptake when compared to those granules which has been Metrizamide purified, but not incubated with Percoll (Table I). Given that granules incubated with Percoll were not exposed to a high G-force centrifugation with Percoll beads and also that possible soluble inhibitors of chromaffin granule Ca^{2+} sequestration had been removed from Percoll by dialysis, neither a physical interaction between granules and Percoll beads, nor a soluble component of Percoll appear responsible for the attenuated ability of chromaffin granules to sequester $^{45}Ca^{2+}$. Given that such an attenuation of $^{45}Ca^{2+}$ uptake into granules by Percoll had been observed, we investigated the dose-dependence of dialysed Percoll on Ca^{2+} uptake, finding that half-maximal inhibition of uptake occurred at 0.60 ± 0.32% Percoll, with maximal inhibition of uptake (> 75%) being seen above 5% Percoll (Fig. 2). Similarly, when the maintenance of intragranular $^{45}Ca^{2+}$ by chromaffin granules which had previously been allowed to take up $^{45}CaCa^{2+}$ was examined, a profound effect of Percoll on chromaffin granule $^{45}Ca^{2+}$ homeostasis was also seen. The incubation of $^{45}Ca^{2+}$-loaded chromaffin granules with dialysed Percoll revealed a dose-dependent loss of granule sequestered $^{45}Ca^{2+}$, with half-maximal loss occurring at 1–2% Percoll, and maximal loss of sequestered $^{45}Ca^{2+}$ occurring at 25% Percoll and involving the loss of 84% of sequestered $^{45}Ca^{2+}$ (Fig. 2, *inset*).

Table I

Ca^{2+} uptake by metrizamide and Percoll-prepared chromaffin granules

Metrizamide prepared granules	Ca^{2+} uptake (nmol/mg of granule protein)
Alone	21.1 ± 1.3
+ 5 μM A23187	337.7 ± 40.2
Incubated with 25% dialysed Percoll	8.0 ± 0.75
Incubated with 25% dialysed Percoll + 5 μM A23187	85.5 ± 4.9

Chromaffin granules were purified by metrizamide density gradient (see section 2) and incubated for 35 min at 37°C in a buffer containing 300 mM sucrose, 25 mM HEPES, pH 7.5, 0.5 mM EGTA, 0.65 mM Ca^{2+}, $^{45}Ca^{2+}$ (10 cpm/pmol) including further additions where specified. The reaction was quenched, and $^{45}Ca^{2+}$ determined by filtration (see section 2). Data shown here are mean ± S.D. (n = 3).

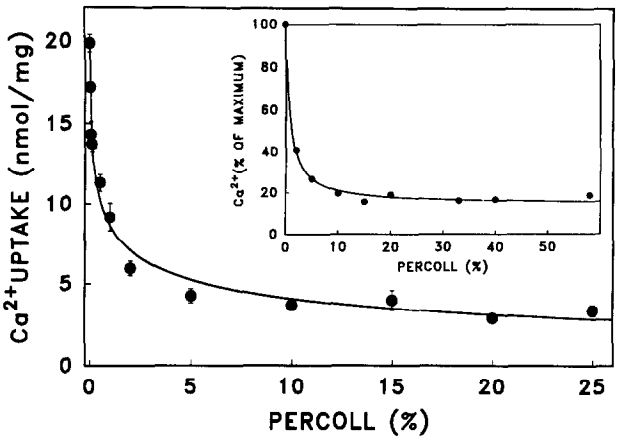

Fig. 2. Dose-dependency of Percoll inhibition of Ca^{2+} uptake and Percoll-induced release from metrizamide-purified chromaffin granules. Chromaffin granules were purified by metrizamide gradient centrifugation and were incubated in a buffer containing 0.3 M sucrose, 0.5 mM EGTA, 25 mM HEPES, pH 7.5, and various concentrations of dialyzed Percoll. Ca^{2+} uptake was initiated by the addition of 0.65 mM $CaCl_2$ (containing 10 cpm/pmol $^{45}Ca^{2+}$), and the reaction was allowed to progress for 45 min at 37°C. After incubation, aliquots were removed and retained $^{45}Ca^{2+}$ was determined by filtration and liquid scintillation spectrometry (section 2). Data shown are mean ± S.D. (n = 3). (*Inset*) Chromaffin granules purified by Metrizamide gradient centrifugation, were incubated in a buffer containing 0.3 M sucrose, 0.5 mM EGTA, 25 mM HEPES, pH 7.5, and Ca^{2+} uptake was initiated by the addition of 0.65 mM $CaCl_2$ (containing 10 cpm/pmol $^{45}Ca^{2+}$). The reaction was allowed to proceed for 45 min at 37°C. Aliquots of this mixture was diluted 1:1 into 0.3 M sucrose, 25 mM HEPES, pH 7.5, which contained where indicated, various concentrations of Percoll, and the reaction was allowed to progress for a further 10 min at 37°C. After this second period of incubation, aliquots were removed and retained $^{45}Ca^{2+}$ was determined by filtration and liquid scintillation spectrometry (section 2). Data shown are representative of three experiments, and are expressed as per cent of granule-sequestered $^{45}Ca^{2+}$ in the absence of Percoll.

In order to further examine the effects of Percoll on $^{45}Ca^{2+}$ uptake into chromaffin granules, the consequences of co-incubating dialysed Percoll with Ca^{2+} ionophore-treated granules were examined. Given that chromaffin granules maintain a large intravesicular to extravesicular proton gradient, and that Ca^{2+} ionophores such as A23187 function as a H^+/Ca^{2+} exchanger, we reasoned that the use of an ionophore would provide information regarding the maximum Ca^{2+} uptake of chromaffin granules, and possibly in part clarify the suppressed uptake of Ca^{2+} seen in Percoll-treated granules. $^{45}Ca^{2+}$ uptake was 337.7 ± 40.2 nmol/mg (Mean ± S.D., n = 3) into Metrizamide purified granules, as a result of concurrent incubation with 5 μM calcium ionophore, A23187 (Table I). Conversely, granules which had been incubated with 25% dialysed Percoll and 5 μM A23187, sequestered 85.5 ± 4.9 nmol $^{45}Ca^{2+}$ per mg of protein (Mean ± S.D., n = 3) (Table I), suggesting that the Percoll substantially disrupts not just the $^{45}Ca^{2+}$ uptake mechanism (mediated by the

Na^+/Ca^{2+} exchange protein), but also possibly elements of the $^{45}Ca^{2+}$ buffering system, or may even compromise granule membrane integrity itself.

It is possible that some of the inhibitory actions of Percoll on Ca^{2+} uptake are due to residual Percoll contamination of the granules after purification. In order to examine this possibility, we compared the Ca^{2+} uptake of chromaffin granules which were derived from the same adrenal tissue under two conditions. We compared the uptake of granules which had been purified through the Metrizamide density gradient twice, with those which were first passed through the Percoll density gradient and then the Metrizamide density gradient. We reasoned that both washing of the Percoll purified granules and passing the Percoll prepared granules through the Metrizamide preparative procedure would remove most of the contaminating Percoll. Indeed, when the level of contaminating Percoll was calculated, the worst case scenario suggested that 0.004% Percoll was present in the extragranular buffer. This concentration of Percoll could not cause significant inhibition of Ca^{2+} uptake (Fig. 2). However, approximately 60% less $^{45}Ca^{2+}$ (10.09 ± 0.22 nmol/mg as opposed to 24.8 ± 2.10 nmol/mg, Mean ± S.D., $n = 3$) was sequestered by Percoll-prepared, Metrizamide-purified granules compared with Metrizamide-prepared granules. This suggests that the Percoll purification of granules causes an irreversible attenuation of their Ca^{2+} sequestering ability.

The question of whether the Percoll-induced inhibition of $^{45}Ca^{2+}$ uptake and induction of release of $^{45}Ca^{2+}$ from Ca^{2+}-loaded chromaffin granules was indeed due to lysis of the granule membrane, was addressed by incubating chromaffin granules with 25% dialysed Percoll for 45 min and determining their catecholamine content. We observed no significant difference ($P < 0.05$) in the catecholamine content of control (3.29 ± 0.21 μmol catecholamines/mg of granule protein: mean ± S.D., $n = 3$) versus Percoll-incubated (2.97 ± 0.33 μmol catecholamines/mg of granule protein: mean ± S.D., $n = 3$) chromaffin granules (Table II). Therefore, it appears that incubation of chromaffin granules with Percoll does not induce a general leakiness in the granule membrane, in that catecholamines do not leak from Percoll purified granules. Thus these effects of Percoll on the Na^+/Ca^{2+} exchange mechanism remain unresolved at present. In the case of the ionophore and Percoll-incubated chromaffin granules, the reduced ability of these granules to accumulate $^{45}Ca^{2+}$ may result from Percoll treatment altering the membrane proton permeability and as a result collapsing the proton gradient which drives A23187-mediated Ca^{2+} uptake. This possibility remains to be tested.

Finally, we emphasize that these inhibitory effects of Percoll on $^{45}Ca^{2+}$ uptake and release have been seen using only three batches of this reagent. However, even though one batch of Percoll we examined was able to produce granules capable of displaying uptake levels approaching those available by other methods (data not shown), the uncertainty about the utility of Percoll for the production of organelles with intact membrane properties is significant. Thus, our findings suggests that this method might not be the one of choice for producing chromaffin granules, or indeed other secretory granules, for studies of their Ca^{2+} uptake and release mechanisms. Conversely, if the means by which Percoll inhibits $^{45}Ca^{2+}$ uptake and stimulates release could be determined, it may help to elucidate the mechanisms of chromaffin granule $^{45}Ca^{2+}$ uptake and release.

Acknowledgements: This work was supported by the Medical Research Council of Canada.

REFERENCES

[1] Kostron, H., Winkler, Geissler, D. and Konig, P. (1977) J. Neurochem. 28, 487–493.
[2] Krieger-Bauer, H. and Gratzl, M. (1982) Biochim. Biophys. Acta 691, 61–70.
[3] Winkler, H., Apps, D.K. and Fischer-Colbrie, R. (1986) Neuroscience 18, 261–290.
[4] Phillips, J.H. and Allison, Y.P. (1977) Neuroscience 2, 147–152.
[5] von Grafenstein, H.R.K. and Powis, D.A. (1989) J. Neurochem. 53, 428–435.
[6] Yoo, S.H. and Albanesi, J.P. (1990) J. Biol. Chem. 265, 13446–13448.
[7] Smith, A.D. and Winkler, H. (1967) Biochem. J. 103, 480–482.
[8] Burgun, C., Martinez de Munoz, D. and Aunis, D. (1985) Biochim. Biophys. Acta 839, 219–227.
[9] Gratzl, M., Krieger-Brauer, H. and Ekerdt, R. (1981) Biochim. Biophys. Acta 355–366.
[10] Morris, S.J. and Schovanka, I. (1977) Biochim. Biophys. Acta 464, 53–64.
[11] Trifaro, J.M. and Dworkind, J. (1970) Anal. Biochem. 34, 403–412.
[12] Meyer, D.I. and Burger, M.W. (1979) J. Biol. Chem. 254, 9854–9859.
[13] Morris, S.J. and Schovanka, I. (1977) Biochim. Biophys. Acta 464, 53–64.
[14] von Euler, U.S. and Lishajko, F. (1961) Acta. Physiol. Scand. 51, 348–356.
[15] Sharman, D.F. (1971) Methods in Neurochemistry (Fried, R., Ed.) Vol. 1, Chapter 3, Marcell Decker, New York.
[16] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.